Induction of Chondroitin Sulfate Lyase Activity in *Bacteroides thetaiotaomicron*

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Chondroitin sulfate lyase (EC 4.2.2.4) was present constitutively at low levels (0.06 to 0.08 U/mg of protein) in cells of *Bacteroides thetaiotaomicron* which were growing on glucose or other monosaccharides. When these uninduced bacteria were incubated with chondroitin sulfate A (5 mg/ml), chondroitin sulfate lyase specific activity increased more than 10-fold within 90 min. Synthesis of ribonucleic acid and of protein was required for induction, and induction was sensitive to oxygen. The disaccharides which resulted from chondroitinase action did not act as inducers, nor did tetrasaccharides or hexasaccharides obtained by digestion of chondroitin sulfate with bovine testicular hyaluronidase. None of these substances was taken up by uninduced cells; they may not have been able to penetrate the outer membrane. The smallest oligomer capable of acting as an inducer was the octasaccharide. Oligomers larger than the octassacharide induced chondroitin lyase activity nearly as well as intact chondroitin sulfate.

Many species of human colonic bacteria require fermentable carbohydrate for growth (5, 9). Since virtually all of the carbohydrate that is available in colons is in the form of polysaccharides, saccharolytic colon bacteria must be able to obtain carbon and energy either directly from polysaccharides or from the products of polysaccharide breakdown by other bacteria. The polysaccharides which enter the colon come from a variety of sources and differ considerably from one another with respect to composition and structure. Some, such as mucins or epithelial cell glycoproteins, are produced by the host. Others, such as plant cell wall polysaccharides, are ingested in the diet and reach the colon because they are not degraded appreciably in the stomach or small intestine. Thus, colonic bacteria which utilize polysaccharides are confronted with a constantly changing mixture of potential carbon sources.

Results of preliminary experiments involving breakdown of polysaccharides by species of *Bacteroides* from human colons indicated that the enzymes responsible for degrading polysaccharides were inducible, since they were produced when the bacteria were grown in a medium containing an appropriate polysaccharide but not produced when bacteria were grown in medium containing monosaccharide components (11, 13, 14). If the enzymes responsible for polysaccharide breakdown are inducible, the survival of the bacteria which rely on these enzymes to obtain carbon and energy depends on how rapidly and under what conditions the necessary enzymes can be produced. To determine what factors are involved in the production of enzymes which degrade polysaccharides, we investigated the conditions under which chondroitin sulfate lyase (EC 4.2.2.4) is produced by *Bacteroides thetaiotaomicron*.

B. thetaiotaomicron (1) is a common isolate from human feces (ca. 10^{10} cells per g [wet weight] [9]). Strains of *B. thetaiotaomicron* are gram negative and obligately anaerobic and are able to utilize a number of polysaccharides, including chondroitin sulfate (1, 5, 15). Chondroitin sulfate is an acidic mucopolysaccharide which is found in tissue (6) and which is probably present in colons due to the extensive sloughing of epithelial cells. When *B. thetaiotaomicron* utilizes chondroitin sulfate, this polymer is first broken into sulfated disaccharides by a chondroitin sulfate lyase which is similar to that produced by *Proteus vulgaris* (10, 19). This enzyme cleaves the $\beta(1 \rightarrow 4)$ glycosidic bond next to the uronic acid by a β-eliminative reaction to produce a disaccharide containing a $\Delta4,5$ uronic acid residue (10, 12, 19). In *B. thetaiotaomicron*, chondroitin sulfate lyase appears to be periplasmic, and there are no extracellular enzymes which degrade chondroitin sulfate (12).

MATERIALS AND METHODS

Organism and growth conditions. *B. thetaiotaomicron* VPI 5482A (NCTC 10852) was obtained from the culture collection of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg. Bacteria were grown in a continuous culture apparatus similar to that described by Kafkewitz et al. (7). The medium was based on the defined

medium of Varel and Bryant (16). Carbonate buffer was replaced with 0.05 M potassium phosphate buffer (pH 7.0). After autoclaving, sterile sodium bicarbonate was added to the medium (final concentration, 0.4%). Glucose was limiting (0.2%), and bacteria were maintained at an optical density at 650 nm of 1.0 to 1.1. This optical density corresponded to a bacterial concentration of 3.5×10^9 colony-forming units per ml. The dilution rate was $0.08 \ h^{-1}$. The volume of the culture vessel was 95 ml, the gas phase was oxygen-free carbon dioxide, and the vessel was maintained at 37°C. Bacterial cultures were equilibrated for 7 to 10 generations before use in the induction experiments. Continuous cultures were used to provide bacteria which were in a reproducible metabolic state before exposure to the inducer. Moreover, since glucose was limiting, the glucose concentration in the continuous culture medium as determined by the Glucostat assay (Sigma Chemical Co.) was always zero. Thus, it was not necessary to wash bacteria before induction in order to remove excess glucose.

Induction experiments. Bacteria were removed anaerobically from the continuous culture vessels. To start the induction process, bacteria were added to stoppered tubes containing chondroitin sulfate A which had been heated and then cooled under nitrogen to prevent oxidation of the bacterial suspension. In experiments to determine whether protein synthesis, RNA synthesis, or DNA synthesis was required for induction, 0.01 ml of a stock solution of chloramphenicol (50 mg/ml) or rifampin (10 mg/ml in methanol) or 0.04 ml of a stock solution of nalidixic acid (50 mg/ml in 0.05 M NaOH) was added to the bacterial suspension immediately before the start of induction. In the case of rifampin, a control to which methanol alone was added was run in parallel. After initiation of induction, 2-ml portions were removed at intervals from the incubation mixture and added to 3 ml of ice-cold 0.05 M potassium phosphate buffer (pH 7.0) to stop the induction process. In some experiments, this bacterial suspension was disrupted by sonication without further treatment and then centrifuged at 15,000 $\times$ g for 10 min at 4°C to remove undisrupted bacteria. In other experiments, when it was necessary to remove spent medium or substances (such as rifampin) which intefered with the enzyme assay, the bacterial suspension was first centrifuged at 15,000 $\times$ g for 10 min at 4°C and then resuspended in potassium phosphate buffer before sonic disruption. Results obtained when diluted suspensions of bacteria were centrifuged and resuspended in buffer before sonication were identical to results obtained when bacteria were disrupted immediately after dilution into phosphate buffer.

Chondroitin sulfate lyase activity released from disrupted bacteria was determined by measuring increases in absorbance at 235 nm (12). Extracellular fluid was also assayed for chondroitin sulfate lyase activity to confirm that no lyase was released from the bacteria during the induction process. One unit of chondroitin sulfate lyase activity was defined as an increase in absorbance at 235 nm of 1.0 U/min at 37°C. Activity was linear throughout the assay period. Protein was measured by the method of Lowry et al. (8), using bovine serum albumin as the standard. All enzyme specific activities reported here were obtained

by averaging results from at least three separate experiments.

To determine the rate at which chondroitin sulfate A disappeared from the medium during induction, 0.5-ml portions of an incubation mixture containing bacteria and 2 mg of chondroitin sulfate A per ml of mixture were removed and added to 4.5 ml of ice-cold water. Bacteria were pelleted by centrifugation at 15,000 $\times$ g and 4°C for 15 min. The concentration of chondroitin sulfate A in the supernatant fluid was then determined by a cetylpyridinium chloride precipitation assay (10), using chondroitin sulfate A as the standard.

Preparation of compounds tested as inducers. Fractions of chondroitin sulfate A having different molecular weights were obtained by chromatography of chondroitin sulfate A on Sephadex G-200 (3, 18), using 1 M NaCl as the eluant. The column dimensions were 2.5 by 80 cm, and the flow rate was 20 ml/h. Fractions of 5 ml were collected, and the concentration of chondroitin sulfate was determined by the carbazole assay for uronic acids (2). The void volume for this column was 180 ml, and the fully included volume was 525 ml. Oligomers of chondroitin sulfate A, which were obtained by digestion of chondroitin sulfate A with bovine testicular hyaluronidase (3), were separated on a column of Sephadex G-50 (fine), using 1 M NaCl as the eluant. The column dimensions were 2.5 by 95 cm, the flow rate was 20 ml/h, and fractions of 4 ml were collected. The void volume was 180 ml, and the total included volume was 460 ml.

Fractions containing the various oligomers were combined and desalted on a smaller Sephadex G-50 column (1.5 by 40 cm), using distilled water as the eluant. When necessary, further purification was accomplished by descending chromatography (3, 12) on Whatman 3MM filter paper in glacial acetic acid-n-butanol-1 N NH₄OH (3:2:2, vol/vol). Chromatograms were developed for at least 36 h. This system could resolve oligomers up to the octasaccharide (DP8). The degree of polymerization (DP) of some oligosaccharides was confirmed by first reducing an oligomer with [³H]borohydride and then comparing the migration distance of this [³H]borohydride-reduced oligomer with the migration distance of an authentic [³H]borohydride-reduced oligomer of known degree of polymerization on strips of Whatman 3MM filter paper (1 by 20 inches [2.54 by 50.8 cm]) in the solvent system described above. After drying, each chromatogram was cut into 0.5-inch (1.27-cm) segments, and the radioactivity in each segment was determined by liquid scintillation counting (3).

The unsaturated sulfated disaccharides ΔDi-4S and ΔDi-6S were obtained by lyase digestions of chondroitin sulfates A and C, respectively, as described in the accompanying paper (12). After separation on paper chromatograms (12), disaccharide bands, which were visualized under UV light, were cut out and eluted with distilled water. A corresponding portion of a chromatogram which contained no disaccharide was also eluted with water. This eluant was used as a control to confirm that residues from the paper or the solvent system did not affect induction.

To determine whether some inducing substance other than ΔDi-4S or ΔDi-6S might be produced by

enzymes from *B. thetaiotaomicron*, chondroitin sulfate A was digested with sonically disrupted cells of *B. thetaiotaomicron* which had been grown either in medium containing chondroitin sulfate or in medium containing glucose as the carbon source. After digestion, low-molecular-weight compounds were separated from undigested chondroitin sulfate by pressure dialysis through an Amicon concentrator equipped with a UM5 filter (Amicon Corp., Elkhart, Ind.). The dialysate was concentrated by flash evaporation at 30°C. This dialysate was free of undigested chondroitin sulfate A, as determined by the cetylpyridinium chloride precipitation assay (10).

Chemicals. Chondroitin sulfate type A (Sigma Chemical Co.) was used in all experiments. This preparation contained about 25% chondroitin sulfate C (12). Authentic standards for the sulfated disaccharides ΔDi-4S and ΔDi-6S and the unsulfated disaccharide ΔDi-0S were obtained from Miles Research Products, Elkhart, Ind. [^{3}H]borohydride-reduced octasaccharide and decasaccharide from chondroitin sulfate A were generous gifts from Janet Glaser, Department of Biochemistry, University of Illinois. The degree of polymerization of these fragments had been determined by the procedure of Glaser and Conrad (3).

RESULTS

Induction of enzyme synthesis. Bacteria growing in a continuous culture with glucose as the sole source of carbohydrate had low but detectable levels of chondroitin sulfate lyase activity (0.06 to 0.08 U/mg of cell protein). This level of activity was constant over dilution times ranging from 0.2 to 0.025 h^{-1}. Similarly constant, low levels of chondroitin sulfate lyase were observed when 11 mM *N*-acetylglucosamine replaced glucose as the limiting carbohydrate. When bacteria from a glucose-limited continuous culture were exposed to chondroitin sulfate A (2 mg/ml), an increase in enzyme specific activity could be detected within 30 min. This increase continued until all of the chondroitin sulfate in the incubation mixture had been used (Fig. 1). Since the increases in enzyme activity were most rapid and reproducible with continuous cultures which had a dilution rate of around 0.08 h^{-1}, this dilution rate was used for all subsequent experiments.

The addition of chloramphenicol (50 μg/ml) or rifampin (25 μg/ml) to a culture before the addition of inducer prevented any increase in enzyme activity. Nalidixic acid (200 μg/ml) did not prevent induction but did reduce the amount of enzyme produced. In mixtures containing nalidixic acid, enzyme specific activity 90 min after addition of the inducer was 50% of that in the control.

The rate at which specific activity increased depended on the concentration of chondroitin sulfate A in the incubation mixture (Fig. 2). At concentrations of less than 0.02 mg of chondroi-
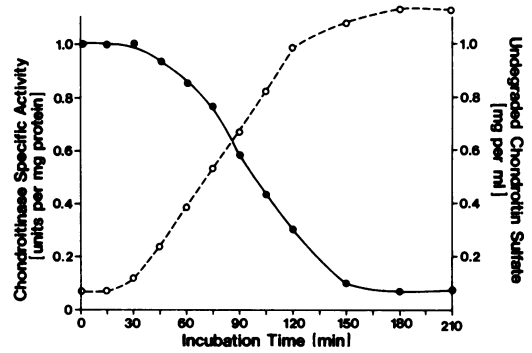

FIG. 1. *Increase in chondroitin sulfate lyase (chondroitinase) specific activity (O) and corresponding disappearance of chondroitin sulfate A from the medium (●) when uninduced cells of B. thetaiotaomicron were incubated with chondroitin sulfate A (final concentration, 1 mg/ml). During the incubation, the optical density at 650 nm of the culture increased from 1.0 to 1.3, indicating that the concentration of cells increased by 30%.*
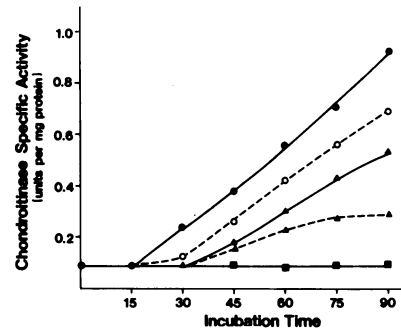

FIG. 2. *Effect of initial chondroitin sulfate A concentration on the rate of chondroitin sulfate lyase (chondroitinase) induction. Symbols: ●, concentrations of 5.0 mg/ml and higher; O, 1.0 mg/ml; ▲, 0.2 mg/ml; △, 0.05 mg/ml; ■, 0.02 mg/ml.*

tin sulfate A per ml, there was no detectable increase in enzyme activity. Between 0.02 and 5.0 mg/ml, the rate of change in specific activity increased with the initial chondroitin sulfate A concentration and the lag period decreased. At concentrations above 5 mg/ml, there was no further change in the rate of induction or the length of the lag period.

In the experiments just described, bacteria were taken directly from the continuous culture vessel and used without further treatment. Anaerobiosis was maintained throughout. When bacteria were harvested by centrifugation and resuspended in 0.05 M potassium phosphate buffer (pH 7.0) before addition of the inducer, no induction occurred (Fig. 3). This was due in part to the centrifugation steps, since centrifu-

gation in tubes which had been flushed and sealed under carbon dioxide followed by suspension in prereduced basal medium resulted in a decrease in the rate of induction (Fig. 3). However, induction was not abolished if anaerobiosis was maintained. Complete loss of inducibility occurred when the induction process was carried out under oxidizing conditions (i.e., when bacteria taken from the continuous culture were bubbled with air for 5 min before addition of the chondroitin sulfate and throughout the induction period). Bubbling with oxygen-free carbon dioxide or nitrogen did not affect induction.

Effect of chondroitin sulfate molecular weight. To determine whether the inducer of chondroitin sulfate lyase synthesis was some low-molecular-weight contaminant of chondroitin sulfate A rather than the polymer itself, chondroitin sulfate A was subjected to chromatography on Sephadex G-200 (Fig. 4). Since chondroitin sulfate A is polydisperse with respect to molecular weight, it eluted from this column in a broad symmetrical peak. Fractions containing material eluting between 180 and 350 ml were pooled, concentrated by flash evaporation, and desalted on a Sephadex G-50 column. When this preparation was used as the inducer, the increase in enzyme activity was identical to that obtained with unfractionated chondroitin sulfate A. Chondroitin sulfate which had been dialyzed for 24 h against three changes of distilled water was similarly effective as an inducer.
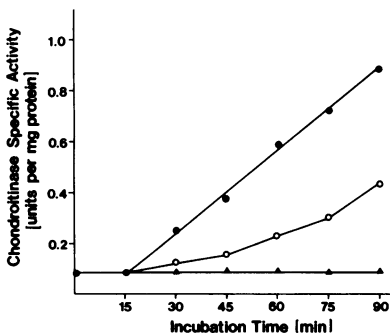

FIG. 4. *Chromatographic profile of a chondroitin sulfate A preparation (heavy solid line). Rechromatography of pooled fractions (indicated by dashed lines) gave profiles with peaks at different elution volumes (thin solid lines), indicating that pooled fractions I, II, and III contained different molecular weight distributions of chondroitin sulfate A. Pooled fractions I, II, and III were equally effective as inducers of chondroitin sulfate lyase activity (see text).*

FIG. 3. *Effect of centrifugation and oxidation on induction. When bacteria were centrifuged anaerobically and suspended in prereduced medium under oxygen-free carbon dioxide, the rate of induction (○) was lower than that in the control (●). Induction was completely abolished by centrifuging and suspending bacteria in 0.05 M phosphate buffer (pH 7.0) or by bubbling cells with air throughout the induction period (▲). Parallel cultures which were bubbled with oxygen-free carbon dioxide or nitrogen rather than air showed increases in chondroitin sulfate lyase (chondroitinase) specific activity which were identical to those of the control (●).*
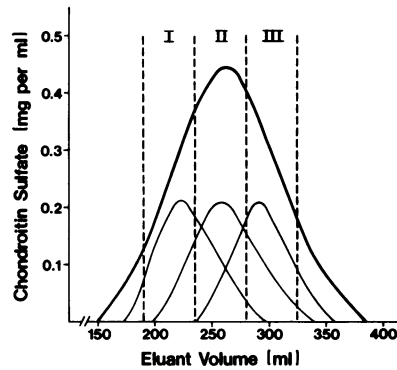
To determine whether fractions of chondroitin sulfate having different molecular weights were equally effective as inducers, fractions from three portions of the broad chondroitin sulfate peak were pooled (Fig. 4, fractions I, II, and III). When these pooled fractions were rechromatographed on Sephadex G-200, they eluted as overlapping peaks with different average elution times (Fig. 4). Accordingly, pooled fractions I, II, and III were assumed to contain different molecular weight distributions. To compare the inducing capabilities of these three size ranges of chondroitin sulfate, enough of each was added to uninduced bacteria to give a final concentration in the reaction mixture of 1 mg of chondroitin sulfate per ml (as determined by the carbazole assay). After 120 min the lyase specific activity in bacteria incubated with pooled fraction I increased by 0.93 U/mg of protein, compared with 0.92 U/mg of protein for fraction II, 0.99 U/mg of protein for fraction III, and 1.02 U/mg of protein for unfractionated chondroitin sulfate A. Since the variation in these determinations was 5 to 7%, the differences were not significant.

Induction of chondroitin sulfate lyase synthesis by components of chondroitin sulfate. The monosaccharide components of chondroitin sulfate A, *N*-acetylgalactosamine and glucuronic acid, supported growth of *B. thetaiotaomicron* but did not induce lyase activity. Several concentrations (0.1, 0.5, 1.0, and 2.0 mM in the final reaction mixture) were tested, but no increase in lyase activity was detected,

even after prolonged incubation (180 min). The unsaturated disaccharides ΔDi-4S and ΔDi-6S, which were obtained by digestion of chondroitin sulfate A and chondroitin sulfate C, respectively, by chondroitin sulfate lyase from *B. thetaiotaomicron*, were tested as inducers at two concentrations (0.2 and 1 mg/ml). No increase in enzyme activity was detected. In contrast to the monosaccharide components, there was no increase in the optical density of the induction mixture with the disaccharides, indicating that the disaccharides did not support growth. Moreover, the concentration of disaccharides in the extracellular fluid, as determined by the carbazole test, did not decrease during the incubation period. Thus, the failure of these disaccharides to act as inducers may have been due to their inability to get past the outer membrane and into the cell. Their failure to induce was not due to contamination by residual solvent from the paper chromatographic purification step, since the eluant from a comparable portion of a chromatogram which had not been streaked with carbohydrate did not inhibit induction by chondroitin sulfate A. When both ΔDi-4S (or ΔDi-6S) and chondroitin sulfate A were added to the reaction mixture at concentrations of 1 mg/ml, the lyase specific activity after 120 min was 10% lower than that observed when chondroitin sulfate A was the only inducer. Thus, the disaccharides had a slightly inhibitory effect on induction by intact chondroitin sulfate A.

To determine whether some inducing substance other than the disaccharides was produced during breakdown of chondroitin sulfate by *B. thetaiotaomicron*, digests obtained by incubating chondroitin sulfate A with disrupted bacteria were tested for the ability to induce lyase activity. One digest was made by using disrupted induced bacteria (i.e., bacteria which had been grown on chondroitin sulfate). Another digest was made by using disrupted uninduced bacteria (i.e., bacteria which had been grown on glucose). Both of these digests contained ΔDi-4S, ΔDi-6S, ΔDi-0S, and N-acetylgalactosamine, as well as other, unidentified, low-molecular-weight substances. The concentrations of ΔDi-4S, ΔDi-6S, ΔDi-0S, and N-acetylgalactosamine were lower in the digest made with the enzyme from uninduced bacteria than in the digest made with the enzyme from induced bacteria. Neither digest induced chondroitin sulfate lyase synthesis.

Since the disaccharides did not induce lyase activity, whereas undegraded chondroitin sulfate did, we reasoned that there must be some oligomer of chondroitin sulfate which is intermediate between the disaccharide and the polymer and which can act as an inducer of lyase

activity. To test this hypothesis, we incubated oligosaccharides ranging from the tetrasaccharide (DP4) to an oligomer containing 16 monomers (DP16) with uninduced cells and measured the increase in chondroitin sulfate lyase activity after 60 and 120 min. These oligomers were obtained by digestion of chondroitin sulfate A by bovine testicular hyaluronidase. Unlike bacterial lyase, this enzyme is a hydrolase; it breaks chondroitin sulfate into a series of fragments, the smallest of which is a tetrasaccharide. These fragments contain an even number of monosaccharides (i.e., DP4, DP6, DP8, etc.). Because the enzyme is a hydrolase, these oligomers do not contain Δ4,5 uronic acid residues.

Incubation of the tetrasaccharide or hexasaccharide with uninduced cells did not result in any increase in enzyme activity (Table 1). Moreover, these substances were not utilized since the optical density of the culture did not increase and the concentration of oligosaccharide in the medium did not decrease during the 120-min incubation period. A small increase in enzyme activity was observed when the octasaccharide was used as the inducer, and larger increases in enzyme activity were observed with larger oligomers (Table 1). In the case of the octasaccharide and larger oligomers, there were corresponding increases in optical density of the culture and decreases in the concentration of inducer in the medium, indicating that the cells were growing and that the inducer was being utilized. The increase in lyase activity obtained with the larger oligomers (DP12 to DP16) was nearly equal to that obtained with undegraded chondroitin sulfate A. The failure of the tetra- and hexasaccharides to act as inducers was not due to the inability of lyase to degrade them. When lyase from disrupted bacteria was incubated with various oligomers, ranging from the tetrasaccharide to the decasaccharide (final concentration, 1.0 mg/ml in the reaction mixture), the enzyme activity was the same regardless of whether the tetrasaccharide, the hexasaccharide, or a larger oligomer was used as the substrate.

The identities of the oligosaccharides which had been identified tentatively as the octasaccharide and the decasaccharide on the basis of their migration rates on paper chromatograms were confirmed by comparing the migration distances of their [³H]borohydride-reduced forms with the migration distances of authentic [³H]borohydride-reduced standards (Fig. 5). The oligosaccharide which had been identified as the decasaccharide was contaminated by a small amount of larger oligosaccharide (Fig. 5). This was not surprising in view of the fact that in this solvent system the decasaccharide barely

TABLE 1. *Ability of oligomers of increasing size to induce chondroitin sulfate lyase synthesis in B. thetaiotaomicron* [a]

Inducer[b]	Tentative identification[c]	Increase in chondroitin sulfate lyase sp act (U/mg of protein) after:		% of inducer remaining in extracellular fluid after 120 min	Increase in optical density at 650 nm after 120 min
		60 min	120 min		
F1	DP4	ND[d]	ND	100	0.02
F2	DP6	ND	ND	100	0.02
F3	DP8	0.03	0.11	98	0.02
F4	DP10	0.15	0.39	82	0.07
F5	DP12	0.29	0.75	69	0.23
F6	DP14	0.36	0.82	64	0.20
F7	DP16	0.38	0.97	64	0.22
Chondroitin sulfate A		0.42	1.10	30	0.30

[a] The concentration of each inducer in the medium was 1 mg/ml, as determined by the carbazole assay.

[b] F1 through F7, Fractions from Sephadex G-50 chromatography of chondroitin sulfate A digested by hyaluronidase. F1 through F4 were further purified by paper chromatography.

[c] Tentative identifications were based on the elution order from Sephadex G-50 and in the case of DP4 through DP10, on migration distances during chromatography on Whatman no. 1 filter paper.

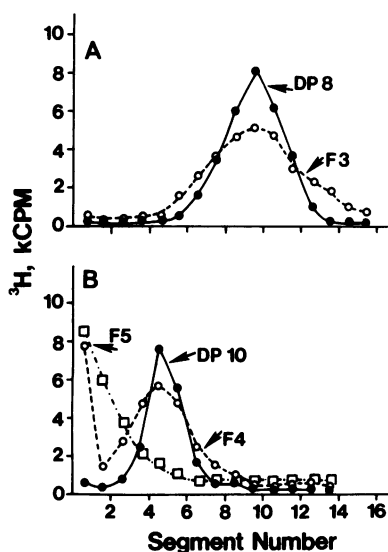[d] ND, Not detectable. The limit of detection was 0.02 U/mg of protein.

FIG. 5. *Confirmation of the tentative identification of fractions 3 (F3) and 4 (F4) as octasaccharide (DP8) and decasaccharide (DP10), respectively. (A) [^{3}H]borohydride-reduced F3 (O) had the same migration distance on a descending Whatman 3MM chromatogram as an authentic [^{3}H]borohydride-reduced octasaccharide (●). (B) [^{3}H]borohydride-reduced F4 (O) comigrated with an authentic [^{3}H]-borohydride-reduced decasaccharide (●). [^{3}H]borohydride-reduced F5 (□) did not migrate away from the origin, indicating that it was a larger oligosaccharide than DP10. Note that F4 contained a small amount of oligosaccharide with a degree of polymerization greater than 10.*

migrated away from the origin even after 48 h and was thus difficult to purify completely by paper chromatographic means. Although the tetrasaccharide and the hexasaccharide did not act as inducers, they did interfere with induction of the lyase by chondroitin sulfate A. When chondroitin sulfate A (1 mg/ml) and either tetra-, hexa-, or octasaccharide (1 mg/ml) were added together to uninduced cells, the lyase activity after 120 min was 0.81 U/mg of protein for the tetrasaccharide, 0.63 U/mg of protein for the hexasaccharide, and 0.54 U/mg of protein for the octasaccharide, compared with 1.07 U/mg of protein when chondroitin sulfate alone was added. Since the variation in these measurements was 5 to 7%, these differences were significant.

DISCUSSION

Synthesis of chondroitin sulfate lyase appears to be inducible in *B. thetaiotaomicron*. Only very low levels of enzyme activity were detected in bacteria growing on glucose or other monosaccharides. However, when these bacteria were incubated with chondroitin sulfate, an increase in lyase specific activity was detectable within 30 min. Since glucose and other fermentable monosaccharides repress lyase induction (Kotarski, unpublished data), the increase in enzyme activity which was observed when bacteria were shifted from glucose to chondroitin sulfate could have been due to derepression rather than induction. However, shifting bacteria from glucose to polysaccharides which are not structurally related to chondroitin sulfate (such as larch arabinogalactan) did not lead to a similar increase in enzyme activity (Salyers, unpublished data).

Chloramphenicol and rifampin both prevented an increase in enzyme activity when bacteria were exposed to chondroitin sulfate, indicating that protein synthesis and RNA synthesis are required for induction. The fact that nalidixic acid, an inhibitor of DNA synthesis, partially inhibited induction may have been due to

secondary effects of this antibiotic on RNA and protein syntheses. The induction process was also sensitive to oxygen. Induction was observed only if anaerobiosis was maintained throughout the induction process. Glass et al. (4) have reported that exposure of *B. thetaiotaomicron* to oxygen shuts off protein synthesis, as measured by incorporation of labeled amino acids into trichloroacetic acid-precipitable material. Since none of the steps involved in the breakdown of chondroitin sulfate by *B. thetaiotaomicron* is sensitive to oxygen (12), the oxygen sensitivity of chondroitin sulfate lyase induction is probably due mainly to a general effect of oxygen on protein synthesis.

Chondroitin sulfate itself, rather than some low-molecular-weight contaminant in the commercial preparation, was the inducer. Sulfated disaccharides, the products of lyase action, did not act as inducers of chondroitinase activity and were not taken up by the bacteria. When *B. thetaiotaomicron* is growing on chondroitin sulfate, the polymer is broken into disaccharides in the periplasmic space. Sulfatases and other degradative enzymes are intracellular (12). Since this indicates that the sulfated disaccharides can be transported across the cytoplasmic membrane once they are inside the periplasmic space, the lack of uptake of the negatively charged disaccharides from the medium is probably due to difficulty in penetrating the outer membrane.

Tetrasaccharides and hexasaccharides, which were obtained by digestion of chondroitin sulfate with testicular hyaluronidase, also did not act as inducers and were not taken up by the bacteria. Since chondroitin sulfate lyase could degrade the tetrasaccharide and the hexasaccharide, as well as the larger oligomers, the failure of these fragments to act as inducers indicates that, like the disaccharides, they were not able to penetrate the outer membrane. The octasaccharide and larger oligomers of chondroitin sulfate did act as inducers, and oligomers containing 10 or more residues were nearly as effective in inducing synthesis of lyase as undegraded chondroitin sulfate. Thus, there may be an outer membrane receptor which is specific for the larger oligomers of chondroitin sulfate. For oligomers larger than the octasaccharide, the ability to induce continued to increase with the number of residues. Since length probably affects the configuration of the polysaccharide chain, such a receptor might have a specificity for structure, as well as for linkage and composition. The tetrasaccharide, the hexasaccharide, and the octasaccharide, although not effective as inducers, interfered with induction by chondroitin sulfate. Accordingly, if there is an outer membrane receptor for chondroitin sulfate and oligomers longer than eight residues, it may also bind the smaller oli-gomers but be unable to bring them into the periplasmic space. Further investigation of the possibility that an outer membrane receptor is needed for utilization of chondroitin sulfate is currently under way in our laboratory.

Induction of chondroitin sulfate lyase synthesis was relatively rapid. Appreciable levels of enzyme activity were detectable within 1 h. The bacteria used for these experiments were growing at a rate of approximately one generation every 9 h. Growth rates of this magnitude or lower are probably typical of the growth rates which are possible for bacteria in colons. Thus, our results indicate that *B. thetaiotaomicron* growing slowly under carbon-limited conditions is capable of inducing enzymes for polysaccharide breakdown within a relatively short time if the concentration of inducer is high enough. The lowest concentration of chondroitin sulfate at which the bacteria produced a measurable increase in enzyme activity was 0.05 mg/ml. Since the concentration of bacteria used in these experiments was about one-fourth the concentration of these organisms in human colons (9), the concentration of chondroitin sulfate in the colon interior would have to be on the order of 0.2 mg/ml or higher to produce a comparable effect. No measurement has been made of the concentration of chondroitin sulfate available to colon bacteria. However, Vercellotti et al. (17) have determined the concentrations of uronic acid and galactosamine in high-molecular-weight carbohydrates from human ileal contents. The concentration of uronic acid was about 1 mg/g (dry weight) or 0.1 mg/ml of intestinal contents. The concentration of galactosamine was two to five times higher. Since mucopolysaccharides are probably a major source of uronic acid and galactosamine, this indicates that the concentration of mucopolysaccharides could be on the order of 0.2 mg/ml in material entering the colon. This may be an underestimate, since Vercellotti et al. (17) were analyzing high-molecular-weight carbohydrates which were solubilized in water at room temperature from lyophilized intestinal contents. Under these conditions, much of the mucopolysaccharide, especially that in aggregates, may not have been solubilized. In any event, chondroitin sulfate-degrading activity can be detected in bacteria obtained directly from human feces (11). Thus, there must be enough chondroitin sulfate in colons to trigger synthesis of the degradative enzymes, although the exact concentration of chondroitin sulfate and the extent to which this concentration varies with time have yet to be determined.

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